PATENT

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for

METHODS AND COMPOSITIONS FOR PRODUCTION OF FLAVONOID AND ISOFLAVONOID NUTRACEUTICALS

by

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BACKGROUND OF THE INVENTION

This application claims the priority of U.S. Provisional Patent Application No. 60/409,447, filed September 10, 2002, the entire disclosure of which is specifically incorporated herein by reference.

1. Field of the Invention

The present invention generally relates to plant genetics. More specifically, the invention relates to methods and compositions for the modulation of flavonoid and isoflavonoid biosynthesis in plants.

2. Description of the Related Art

Isoflavonoid natural products are limited primarily to leguminous plants, where they function as pre-formed or inducible antimicrobial or anti-insect compounds, as inducers of the nodulation genes of symbiotic Rhizobium bacteria, and as allelopathic agents (Dixon, 1999). However, much attention is now being focused on the simple isoflavones daidzein and genistein in view of many reports linking their dietary consumption to a range of potential health benefits (Barnes, 1998; Barnes, 1996; Adlercreutz. and Mazur, 1997; Adlercreutz, 1998; Dixon, 1999; Dixon, 2002). The major dietary sources of these compounds for humans are soybean seed products, which are rich in daidzein and genistein, and chickpea seeds, which also contain biochanin A (4'-*O*-methylgenistein). The structures of these isoflavones are shown in FIG. 1.

Epidemiological studies have demonstrated a link between consumption of soy isoflavones and reduced risks of breast and prostate cancers in humans (Messina *et al.,* 1994; Adlercreutz, 1998). Genistein and biochanin A exhibit chemopreventative activity against chemically-induced cancers in a number of mammalian cell model systems (Fotsis *et al.,* 1995; Lamartiniere *et al.,* 1995; Rauth *et al.,* 1997; Uckun *et al.,* 1995; Yanagihara *et al.,* 1993). Furthermore, isoflavones have been shown to possess other health promoting activities, including potential chemoprevention of osteoporosis and cardiovascular disease

(Anderson and Garner, 1998; Draper *et al.*, 1997; Tikkanen *et al.*, 1998; Wagner *et al.*, 1997). In addition to isoflavonoids, many other flavonoid-derived compounds have been ascribed health-promoting activity. These include flavonols, such as quercetin, that occur at significant levels in leaves and fruit of many plant species and that have high antioxidant activity (Rice-Evans and Miller, 1996).

Isoflavonoids are formed by a branch of the flavonoid biosynthetic pathway, and originate from a central flavanone intermediate that is ubiquitously present in plants. For entry into the isoflavonoid pathway, the flavanone naringenin undergoes migration of the B-ring from the 2- to the 3-position followed by hydroxylation at the 2-position catalyzed by a microsomal cytochrome P450 enzyme, CYP93C1 (2-hydroxyisoflavanone synthase or 2-HIS, also commonly termed isoflavone synthase (IFS) (FIG.1). The resulting 2-hydroxyisoflavanone then undergoes dehydration to yield the corresponding isoflavone genistein (Hakamatsuka *et al.*, 1990; Kochs and Grisebach, 1986), as shown in FIG. 1. The dehydration reaction can take place non-enzymatically *in vitro* under acid conditions (Kochs and Grisebach, 1986), although an enzyme has been purified from cell cultures of *Pueraria lobata* that can catalyze this reaction (Hakamatsuka *et al.*, 1998). This dehydratase appears to be closely associated with the microsomal aryl migration enzyme (Hakamatsuka *et al.*, 1998).

cDNA clones that encode IFS have been characterized from soybean and other legumes (Akashi *et al.*, 1999; Jung *et al.*, 2000; Steele *et al.*, 1999). The soybean enzyme is classified as CYP93C1v2. When expressed in insect cells, it converts the flavanones liquiritigenin and naringenin directly to their corresponding isoflavones daidzein and genistein in the presence of NADPH (Steele *et al.*, 1999), as shown in FIG. 1. It is not clear whether dehydration of the putative 2-hydroxyisoflavanone intermediate occurs on the enzyme, or results from an endogenous dehydratase activity present in the insect cell microsomes. The aryl migration enzyme from licorice (*Glycyrrhiza echinata*) has been shown to produce a 2-hydroxyisoflavanone from a flavanone when expressed in yeast (Akashi *et al.*, 1999).

There have been few reports to date on the introduction of new natural product pathways into plants through genetic manipulation. An important recent example is the

introduction of the provitamin A pathway into rice, a process that involved the introduction of three genes (Ye *et al.*, 2000). The soybean IFS has been introduced into the model crucifer *Arabidopsis thaliana* by *Agrobacterium*-mediated transformation (Jung *et al.*, 2000; International Application No. PCT/US00/05915, filed March 8, 2000) and into tobacco and corn by *Agrobacterium* or biolistic bombardment methods, respectively (Yu *et al.*, 2000). These studies have shown the production of low levels of genistein (up to a maximum of around 40 nmol/g fresh weight, but generally less) after hydrolysis of potential genistein glyco-conjugates that may have been formed in the transgenic plants. The nature of the potential glyco-conjugates has not been determined. While these studies confirm production of isoflavones in transgenic plants, it would be advantageous to increase the amount of flavonoid natural products produced by the transgenic plants to provide improved bioactivity for dietary health-promotion.

The key biosynthetic reactions leading to the formation of flavanone are catalyzed by chalcone synthase (CHS) and chalcone isomerase (CHI). CHS is a homodimeric polyketide synthase that forms 2',4,'4',6'-tetrahydroxychalcone (naringenin chalcone) from three molecules of malonyl coenzyme A and one molecule of 4-coumaroyl CoA according to the reaction shown in FIG. 1. It is often encoded by a multigene family in legumes (Junghans *et al.*, 1993), and many *CHS* genes have been cloned from a wide number of plant species, including alfalfa (Schröder, 1997).

CHI is a monomeric enzyme that very efficiently catalyzes the isomerization of naringenin chalcone to its corresponding flavanone, naringenin (4', 5, 7-trihydroxy-flavanone), as depicted in FIG. 1. This reaction can also occur spontaneously at alkaline pH, although without a stereochemical direction (CHI specifically catalyzes formation of (-) flavanone). For this reason, few plants accumulate naringenin chalcone. CHI from certain legumes can also act on 2', 4, 4'-trihydroxychalcone (isoliquiritigenin), to form the corresponding flavanone liquiritigenin (4', 7, dihydroxy-flavanone) (Dixon *et al.*, 1988), whereas the enzyme from most non-legumes does not appear to have this activity. CHI genes have now been cloned from several species, including alfalfa (McKhann and Hirsch, 1994). In view of its high activity level in many plant tissues relative to the activity level of CHS,

CHI has not heretofore been thought of as a rate-determining enzyme for flavanone formation. However, it has recently been shown that CHI is rate limiting for flavonoid biosynthesis in tomato fruit peel (Muir *et al.*, 2001).

However, in that particular study, expression of the 35S promoter driven petunia CHI transgene did not increase flavonol levels in the flesh of the fruit, or in the leaves. The peel of wild-type tomato fruit unusually contains high levels of naringenin chalcone but very low levels of flavonols, suggesting limitation of CHI activity and explaining the approximately 80-fold increase in flavonols following expression of the CHI transgene (Muir *et al.*, 2001). Such an increase was not observed when maize CHI was over-expressed in wild-type *Arabidopsis* and, in fact, such plants appeared to have reduced levels of anthocyanins (Dong *et al.*, 2001).

The foregoing studies have provided a further understanding of the metabolism of plant secondary metabolism. However, the prior art has failed to provide techniques for the application of this understanding to the creation of plants having valuable new characteristics. What are thus needed are practical techniques for the production of novel plants with improved phenotypes and methods for the use thereof. Such techniques may allow the creation and use of plants with improved nutritional quality, thereby benefiting both human and animal health and representing a substantial benefit in the art.

SUMMARY OF THE INVENTION

In one aspect, the invention provides a method of increasing isoflavonoid biosynthesis in a plant comprising: a) down-regulating flavanone 3-hydroxylase in said plant; and b) up-regulating isoflavone synthase and/or the production of a substrate thereof in said plant. The plant may comprise a mutant flavanone 3-hydroxylase gene exhibiting a loss of function with respect to a flavanone 3-hydroxylase gene lacking said mutation. In one embodiment of the invention, isoflavone synthase is upregulated in the plant. Such upregulating may comprise, in certain embodiments, introducing a transgene into the plant encoding isoflavone synthase into said plant. The transgene may be introduced by any method, including genetically transforming said plant or a parent plant of any previous generation of said plant with said

transgene. In certain embodiment of the invention, the isoflavone synthase comprises the polypeptide sequence of SEQ ID NO:2.

In further embodiments of the invention, a method of increasing isoflavonoid biosynthesis may comprise up-regulating chalcone isomerase in said plant, including the polypeptide sequence encoded by SEQ ID NO:3. Such up-regulating may comprise introducing a transgene encoding said chalcone isomerase into said plant by any method, including genetically transforming said plant or a parent plant of any previous generation of said plant with said transgene. The method may also comprise introducing a transgene encoding the PAP1 gene into said plant. In still further embodiments of the invention, the method may comprise up-regulating chalcone synthase in said plant, for example, where chalcone synthase comprises the polypeptide sequence encoded by SEQ ID NO:5 or SEQ ID NO:6.

In one embodiment of the invention, the method of increasing isoflavonoid biosynthesis, down-regulating flavanone 3-hydroxylase comprises introducing a selected DNA into said plant comprising an antisense nucleotide comprising from about 20 or more nucleotides complementary to a gene encoding flavanone 3-hydroxylase. In certain embodiments of the invention, the antisense oligonucleotide comprises from about 20 to about 1242 nucleotides complementary to the nucleic acid sequence of SEQ ID NO:7, from about 20 to about 815 nucleotides complementary to the nucleic acid sequence of SEQ ID NO:10 or from about 20 to about 5586 nucleotides complementary to nucleotides 82850-88437 of SEQ ID NO:8. In still further embodiments, the antisense oligonucleotide is further defined as comprising from about 20 to about 780 nucleotides complementary to nucleotides 82850-83062, 83159-83406, 86908-87232, and/or 87801-88437 of SEQ ID NO:8, or is further defined as comprising from about 20 to about 1021 nucleotides complementary to nucleotides 82850-83062, 83159-83406, 86908-87232, and/or 87801-88043 of SEQ ID NO:8. The antisense oligonucleotide may be introduced by any method, including genetically transforming said plant or a parent plant of any previous generation of said plant with said selected DNA.

The method of increasing isoflavonoid biosynthesis in a plant may be carried out on any plant. In one embodiment of the invention, the plant is a monocotyledonous plant. Examples of monocotyledonous plants include wheat, maize, rye, rice, oat, barley, turfgrass, sorghum, millet and sugarcane. The plant may also be a dicotyledonous plant. Examples of dicotyledonous plants include tobacco, tomato, potato, soybean, cotton, canola, alfalfa, sunflower, and cotton.

In another aspect, the invention provides a transgenic plant stably transformed with: a) a first selected DNA comprising a nucleic acid encoding an antisense oligonucleotide operably linked to a promoter functional in said plant, wherein said antisense oligonucleotide comprises from about 20 to about 1242 nucleotides complementary to the nucleic acid sequence of SEQ ID NO:7, from about 20 to about 815 nucleotides complementary to the nucleic acid sequence of SEQ ID NO:10 or from about 20 to about 5586 nucleotides complementary to nucleotides 82850-88437 of SEQ ID NO:8; and b) a second selected DNA comprising an isoflavone biosynthesis-coding sequence operably linked to a promoter functional in said plant, wherein the coding sequence encodes a polypeptide selected from the group consisting of: the polypeptide of SEQ ID NO:2, the polypeptide encoded by SEQ ID NO:3, the polypeptide encoded by SEQ ID NO:5 and the polypeptide encoded by SEQ ID NO:6.

The first selected DNA and/or said second selected DNA may comprise an enhancer, plasmid DNA, and/or a sequence encoding a signal peptide. In one embodiment of the invention, the transgenic plant is further defined as a fertile R_0 transgenic plant. In another embodiment, the plant is further defined as a progeny plant of any generation of a fertile R_0 transgenic plant, wherein said transgenic plant has inherited said first selected DNA from said R_0 transgenic plant. In yet another embodiment, the transgenic plant is further defined as a progeny plant of any generation of a fertile R_0 transgenic plant, wherein said transgenic plant has inherited said second selected DNA from said R_0 transgenic plant. The transgenic plant may also have inherited said first and said second selected DNA from said R_0 transgenic plant. The first selected DNA and said second selected DNA may also have been transformed into said plant or a progenitor thereof on a single transformation construct.

In certain embodiments of the invention, the transgenic plant of claim 21, wherein the antisense oligonucleotide is further defined as comprising from about 20 to about 780 nucleotides complementary to nucleotides 82850-83062, 83159-83406, 86908-87232, and/or 87801-88437 of SEQ ID NO:8. The antisense oligonucleotide may also be further defined as comprising from about 20 to about 1021 nucleotides complementary to nucleotides 82850-83062, 83159-83406, 86908-87232, and/or 87801-88043 of SEQ ID NO:8.

In yet another aspect, the invention provides a seed of a plant of the invention, wherein said seed comprises said first selected DNA and said second selected DNA.

In still yet another aspect, the invention provides a method of making food for human or animal consumption comprising: (a) obtaining the plant of claim 21; (b) growing said plant under plant growth conditions to produce plant tissue from the plant; and (c) preparing food for human or animal consumption from said plant tissue. In the method, preparing food may comprise harvesting said plant tissue. In certain embodiments, the food may be starch, protein, meal, flour or grain.

In still yet another aspect, the invention provides a method of producing a nutraceutical composition comprising (a) obtaining a plant in accordance with the invention; (b) growing said plant under plant growth conditions to produce plant tissue from the plant; and (c) preparing a nutraceutical composition for human or animal consumption from said plant tissue.

In still yet another aspect, the invention provides a method of inhibiting the initiation and promotion of a mammalian cell to a premalignant or malignant state in a mammal comprising: (a) obtaining the plant in accordance with the invention; (b) growing said plant under plant growth conditions to produce plant tissue from the plant; (c) preparing a nutraceutical composition for human or animal consumption from said plant tissue; and (d) administering a therapeutically effective amount of the nutraceutical composition to the mammal. In one embodiment of the invention, the mammal is a human. In further embodiments, administering is oral or topical.

In still yet another aspect, the invention provides a method of inhibiting the onset of cardiovascular disease in a mammal comprising: (a) obtaining a plant in accordance with the invention; (b) growing said plant under plant growth conditions to produce plant tissue from the plant; (c) preparing a nutraceutical composition for human or animal consumption from said plant tissue; and (d) administering a therapeutically effective amount of the nutraceutical composition to the mammal. In the method, the mammal may be a human. In one embodiment of the invention, administering is oral and/or topical.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein:

FIG. 1: Depicts the reactions catalyzed by chalcone synthase (CHS) and chalcone isomerase (CHI) and the formation of isoflavones from flavanones. Daidzein (R = H) is produced from liquiritigenin, and genistein (R = OH), from naringenin. Naringenin is the product of the action of chalcone isomerase (CHI) on 2',4,4',6'-tetrahydroxychalcone, formed from malonyl CoA and 4-coumaroyl CoA by chalcone synthase (CHS). Formation of liquiritigenin requires the activity of a chalcone reductase (CHR) that co-acts with chalcone synthase (CHS) to form 2',4,4'-trihydroxychalcone followed by the action of chalcone isomerase (CHI). CHI from alfalfa is active against both isoliquiritigenin (2',4,4'-trihydroxychalcone) and naringenin chalcone (2',4,4',6'-tetrahydroxychalcone), whereas CHI from *Arabidopsis* is only active against naringenin chalcone.

FIG. 2A-2D: Depicts HPLC profiles of flavonoids from leaf extracts of wild-type (A) and alfalfa CHI-expressing (B) *Arabidopsis*. Profiles of flavonoids from wild-type (C) and alfalfa CHI-expressing (D) *Arabidopsis* leaves are shown following digestion of the extracts with β-glucosidase. The compounds are: a, Rha-Gluc-Rha-quercetin; b, Rha-Gluc-Rha-kaempferol; c, Rha-Rha-quercetin; d, Rha-Rha-kaempferol; e, Gluc-Rha-quercetin; f, Rutin;

g, h and i, unknown quercetin conjugates; l, kaempferol conjugate; n, kaempferol; j, k and m, quercetin conjugates.

FIG. 3A-3D: Depicts HPLC profiles of flavonoids/isoflavonoids in *Arabidopsis* leaves of a wild-type (Ctrl) plant, or plants expressing soybean IFS (2-HIS 15b), alfalfa CHI (CHI) or progeny of a cross expressing both IFS and CHI (CHI/2-HIS 15b). Leaf extracts were hydrolyzed with HCl prior to chromatography. Compounds were quercetin and a quercetin derivative (Q1, Q2), kaempferol (K), and genistein (G).

FIG. 4A-4D: Depicts HPLC profiles of hydrolyzed extracts from leaves of the Arabidopsis *tt6/tt3* mutant and wild-type Landsberg Erecta (Ler) with and without expression of soybean IFS (T_3 generation). (A) *tt6/tt3*. (*B*) *tt6/tt3* expressing IFS. (C) Ler wild-type. (D) Ler expressing IFS. The compounds are: G, genistein; K, kaempferol; Q, quercetin. FIG. 4E-4F depicts levels of flavonols (E) and genistein (F) in individual T_3 plants of Ler (8-designations) and *tt6/tt3* (9-designations) expressing IFS. Open bars, quercetin; filled bars, kaempferol.

FIG. 5A-C: Depicts HPLC traces of acid-hydrolyzed leaf extracts from MtIFS-overexpressing line C22 (A) and vector control line C11 (B). Peaks 1 and 2 were not observed in control lines and were identified as genistein and biochanin A, respectively by comparing UV spectra and retention times to authentic standards and by LC-MS analysis. The peak labeled A was identified as apigenin. (C) Depicts the amount of genistein present in leaf extracts of 42 independent MtIFS-overexpressing lines.

FIG. 6A-F: Depicts HPLC traces of unhydrolyzed leaf extracts of MtIFS-overexpressing line C22 (A) and vector control line C11 (B). Peaks with UV spectra similar to genistein and not present in the control extracts are numbered 1-5. (C-D) Depicts HPLC traces of leaf extracts of line C22 (C) and control line C11 (D) after digestion with β-glucuronidase. Peaks 1 and 4 were identified as glucose-genistein and glucose-biochanin A, respectively by LC-MS analysis. The peak labeled A was identified as apigenin. (E-F) Depicts HPLC traces of leaf extracts of line C22 (E) and control line C11 (F) after digestion

with β-glucosidase. This enzyme preparation also contained significant β-glucuronidase activity. Peaks labeled G and B were identified as genistein and biochanin A, respectively.

DETAILED DESCRIPTION OF THE INVENTION

The invention overcomes the deficiencies of the prior art by providing methods and compositions for producing plants with enhanced isoflavonoid biosynthesis. Although production of flavonoid metabolites to increase flavonoid precursors can be achieved, for example, by expressing a chalcone isomerase, high concentrations of isoflavonoids have not been achieved. For example, transformation of *Arabidopsis* with soybean isoflavone synthase (IFS), which is also known as 2-hydroxyisoflavanone synthase (2-HIS), in the absence of an introduced 2-hydroxyisoflavanone dehydratase or soybean cytochrome P450 reductase results in production of low levels of genistein in the leaves. *Arabidopsis* appears to glycosylate this new natural product in an identical manner to its endogenous flavonols, namely by conjugation to the sugars rhamnose and/or glucose. *Arabidopsis* plants expressing both a soybean IFS gene and an alfalfa CHI gene do not, however, produce higher amounts of genistein than plants expressing the IFS alone. Thus, the production of antioxidant flavonoids in transgenic plants can be significantly increased by expression of the alfalfa CHI transgene, but other strategies are necessary to obtain significant improvement in isoflavone accumulation.

However, in accordance with the present invention, high levels of isoflavonoid production may be achieved in plants through down-regulation of flavanone 3-hydroxylase (F3H). That is, it has been found that the major problem in previous attempts at engineering isoflavone biosynthesis is the partition of flux between flavonol and isoflavone biosynthesis. This occurs at the level of the flavanone naringenin, which is substrate for both IFS and flavanone 3-hydroxylase (F3H). It has been proposed that a physical complex exists involving CHS, CHI and F3H (Winkel-Shirley, 1999), but the implication with respect to engineering isoflavone biosynthesis has not been known.

The inventors have shown that introduction of isoflavone synthase into plants in which the flavanone 3-hydroxylase is down-regulated results in significant improvement to the levels of accumulation of the isoflavone genistein. This can be coupled with increasing flux into the production of naringenin to result in even further enhancement of isoflavone production. The present invention thus provides for transformation of plants or plant cells with an isoflavone synthase (2-HIS) gene to produce isoflavonoids or glycosides of isoflavonoids. Such plants or plant cells may comprise a down-regulated flavanone 3-hydroxylase relative to other plants of the same variety. Such down-regulation may comprise, for example, use of a naturally-occurring or induced mutant flavanone 3-hydroxylase allele, as well as using antisense or other technology. The down-regulation may comprise complete elimination of expression of flavanone 3-hydroxylase or may comprise a partial decrease in expression.

The present invention also provides for transformation of plants or plant cells with a chalcone isomerase gene to produce increased levels of flavonoid natural products. The present invention provides for transformation of a plant lacking expression of flavanone 3-hydroxylase with IFS in order to produce high accumulation of isoflavones. The invention provides for co-transformation of plants or plant cells lacking flavanone 3-hydroxylase with an isoflavone synthase gene and/or a chalcone isomerase gene to produce isoflavonoids. The invention also provides for other approaches for the generation of transgenic plants expressing an isoflavone synthase gene with parallel over-expression of chalcone isomerase, or any other gene that will increase flux into production of naringenin, and down-regulation of flavanone 3-hydroxylase or any other gene that will block flux into flavonoid biosynthesis downstream of naringenin. Such plants include, for example, vegetables, grains, and fruit, both dicots and monocots, including but not limited to alfalfa, soybean, tomato, lettuce, tobacco, corn, maize, cotton, squash, beans and other legumes, melons, broccoli and other cole crops, stone fruits, citrus fruits, and strawberries. As used herein, unless otherwise stated, the term "plant" or "progeny" includes plant parts, plant tissue, plant cells, plant protoplasts, plant cell tissue cultures from which plants can be regenerated, plant calli, plant clumps, explants, plant cells that are intact in plants, or parts of plants, such as embryos, pollen, ovules, flowers, capsules, stems, leaves, seeds, roots, root tips, and the like.

The methods of producing isoflavonoids or flavonoid natural products from transgenic plants or cells from transgenic plants provided herein presents advantages over production methods which utilized nontransgenic plants in which isoflavonoids or flavonoid natural products occur naturally. The present invention expands the production of isoflavonoids or flavonoid natural products to a wide variety of edible plants, resulting in (1) increased sources for the procurement of isoflavonoids or flavonoid natural products; (2) a larger growing region for isoflavonoid-producing or flavonoid natural product-producing plants which potentially leads to increased production; (3) reduction in seasonal and/or climate limitations associated with nontransgenic isoflavonoid-producing or flavonoid natural product-producing plants; and (4) increased consumer satisfaction.

In the present invention, a plant can be transformed with an isoflavone synthase cDNA or genomic clone; a chalcone isomerase cDNA or genomic clone; or co-transformed with an isoflavone synthase cDNA or genomic clone and a chalcone isomerase cDNA or genomic clone, as well as any synthetic coding sequences of these nucleic acids. In the present invention, plants or plant cells with reduced flavanone 3-hydroxylase expression can be mutant, or generated by antisense, gene silencing (sense suppression), RNAi or other recombinant technologies known to those skilled in the art for down-regulating the expression and/or activity of a given enzyme.

Transgenically produced isoflavonoids or flavonoid natural products may be administered orally by directly ingesting the transgenic plant. Alternatively, the transgenically produced isoflavonoids or flavonoid natural products can be isolated from transgenic plants to be used as a crude extract or purified compound. Administration of transgenically produced isoflavonoids or flavonoid natural products to humans or animals provides enhanced pharmaceutical and nutraceutical effects, including but not limited to anticancer, anti-osteoporosis, anti-oxidant and cardiovascular benefits.

Transformation methods useful in the present invention include but are not limited to *Agrobacterium tumefaciens*-mediated transformation by vacuum infiltration or leaf disc transformation, biolistic particle bombardment, pollen transformation, protoplast electroporation, or permeabilization. Likewise, several methods known in the art can be used

to distinguish the progeny exhibiting stable inheritance of the transgene. For transgenic plants wherein the transgenic cassette contains a gene coding for a visible phenotypic change, the selection can be based upon visual examination of the progeny. For plant transformations involving a selectable marker gene, the appropriate selectable agent can be applied to the plants to select the transformants. Optionally, Southern blot analysis or PCR analysis can be used to verify the presence of the transferred gene in the genome of the transformed plants. RNA gel blot analysis, RT-PCR, or similar techniques can be used to verify the transcription of the transferred gene in transformed tissues. Progeny which are stably transformed and successfully accumulating isoflavonoids or flavonoid natural products can be identified by chemically analyzing the plant tissues for their presence, using chemical methods including but not limited to organic extraction followed by high pressure liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC/MS), gas chromatography (GC), gas chromatography-mass spectrometry (GC-MS), or capillary electrophoresis (CE).

I. Plant Transformation Constructs

Certain embodiments of the current invention concern plant transformation constructs. For example, one aspect of the current invention is a plant transformation vector comprising one or more flavonoid and/or isoflavonoid biosynthesis gene. Exemplary coding sequences for use with the invention include chalcone isomerase (SEQ ID NO:3 and SEQ ID NO:4); chalcone synthase (SEQ ID NO:5 and SEQ ID NO:6) and isoflavone synthase (SEQ ID NO:1). In certain embodiments, antisense flavanone 3-hydroxylase sequences are employed with the invention. Exemplary flavanone 3-hydroxylase nucleic acids include at least 20, 40, 80, 120, 300 and up to the full length of the nucleic acid sequences of SEQ ID NO:7 (*Arabidopsis thaliana*; Genbank Accession No. AJ295587.1), SEQ ID NO:8 (rice; Genbank Accession No. AC092697; gene: 82850-88437; mRNA: 82850-83062, 83159-83406, 86908-87232, and 87801-88437; coding sequence: 82850-83062, 83159-83406, 86908-87232, and 87801-88043) and SEQ ID NO:10 (*Juglans nigra*; Genbank Accession No. AJ278457) may be used. Examples of certain such sequences, each of which may be used, for example, as antisense oligonucleotides, include the nucleic acid sequences of nucleotides 82850-82870, 82850-82890, 82850-82950, 82850-83062, 83159-83179, 83159-83259, 86908-86928,

86908-87008, 86908-87202, 87801-87821, 87801-87901 and/or 87801-88001 of SEQ ID NO:8. Other exemplary sequences include the sequences of nucleotides 1-20, 1-40, 1-100, 100-300, 1-300, 1-500, 1-800 and 1-1242 of SEQ ID NO:7, as well as 1-20, 1-40, 1-100, 100-1300, 1-800 and 1-815 of SEQ ID NO:10.

In certain embodiments of the invention, coding sequences are provided operably linked to a heterologous promoter, in either sense or antisense orientation. Expression constructs are also provided comprising these sequences, as are plants and plant cells transformed with the sequences. The construction of constructs which may be employed in conjunction with plant transformation techniques using these or other sequences according to the invention will be known to those of skill of the art in light of the present disclosure (see, for example, Sambrook *et al.,* 1989; Gelvin *et al.,* 1990). The techniques of the current invention are thus not limited to any particular nucleic acid sequences.

One important use of the sequences provided by the invention will be in the alteration of plant phenotypes by genetic transformation with sense or antisense flavonoid and/or isoflavonoid biosynthesis genes. The flavonoid and/or isoflavonoid biosynthesis gene may be provided with other sequences. Where an expressible coding region that is not necessarily a marker coding region is employed in combination with a marker coding region, one may employ the separate coding regions on either the same or different DNA segments for transformation. In the latter case, the different vectors are delivered concurrently to recipient cells to maximize cotransformation.

The choice of any additional elements used in conjunction with the flavonoid and/or isoflavonoid biosynthesis coding sequences will often depend on the purpose of the transformation. One of the major purposes of transformation of crop plants is to add commercially desirable, agronomically important traits to the plant. As isoflavonoids are known to confer many beneficial effects on health, one such trait is increased biosynthesis of isoflavonoids. Concomitant increases in flavonoid production may also be beneficial and could be achieved by increasing expression of precursors of flavonoid and isoflavonoid

compounds. Alternatively, plants may be engineered to decrease synthesis of flavonoids and/or isoflavonoids.

Vectors used for plant transformation may include, for example, plasmids, cosmids, YACs (yeast artificial chromosomes), BACs (bacterial artificial chromosomes) or any other suitable cloning system, as well as fragments of DNA therefrom. Thus when the term "vector" or "expression vector" is used, all of the foregoing types of vectors, as well as nucleic acid sequences isolated therefrom, are included. It is contemplated that utilization of cloning systems with large insert capacities will allow introduction of large DNA sequences comprising more than one selected gene. In accordance with the invention, this could be used to introduce genes corresponding to the entire flavonoid and/or isoflavonoid biosynthetic pathway into a plant. Introduction of such sequences may be facilitated by use of bacterial or yeast artificial chromosomes (BACs or YACs, respectively), or even plant artificial chromosomes. For example, the use of BACs for *Agrobacterium*-mediated transformation was disclosed by Hamilton *et al.* (1996).

Particularly useful for transformation are expression cassettes which have been isolated from such vectors. DNA segments used for transforming plant cells will, of course, generally comprise the cDNA, gene or genes which one desires to introduce into and have expressed in the host cells. These DNA segments can further include structures such as promoters, enhancers, polylinkers, or even regulatory genes as desired. The DNA segment or gene chosen for cellular introduction will often encode a protein which will be expressed in the resultant recombinant cells resulting in a screenable or selectable trait and/or which will impart an improved phenotype to the resulting transgenic plant. However, this may not always be the case, and the present invention also encompasses transgenic plants incorporating non-expressed transgenes. Preferred components likely to be included with vectors used in the current invention are as follows.

A. Regulatory Elements

Exemplary promoters for expression of a nucleic acid sequence include plant promoter such as the CaMV 35S promoter (Odell *et al.,* 1985), or others such as CaMV 19S (Lawton *et*

al., 1987), *nos* (Ebert *et al.*, 1987), Adh (Walker *et al.*, 1987), sucrose synthase (Yang & Russell, 1990), a-tubulin, actin (Wang *et al.*, 1992), *cab* (Sullivan *et al.*, 1989), PEPCase (Hudspeth and Grula, 1989) or those associated with the R gene complex (Chandler *et al.*, 1989). Tissue specific promoters such as root cell promoters (Conkling *et al.*, 1990) and tissue specific enhancers (Fromm *et al.*, 1986) are also contemplated to be particularly useful, as are inducible promoters such as ABA- and turgor-inducible promoters. In one embodiment of the invention, the native promoter of a flavonoid and/or isoflavonoid biosynthesis gene is used.

The DNA sequence between the transcription initiation site and the start of the coding sequence, *i.e.*, the untranslated leader sequence, can also influence gene expression. One may thus wish to employ a particular leader sequence with a transformation construct of the invention. Preferred leader sequences are contemplated to include those which comprise sequences predicted to direct optimum expression of the attached gene, *i.e.*, to include a preferred consensus leader sequence which may increase or maintain mRNA stability and prevent inappropriate initiation of translation. The choice of such sequences will be known to those of skill in the art in light of the present disclosure. Sequences that are derived from genes that are highly expressed in plants will typically be preferred.

It is contemplated that vectors for use in accordance with the present invention may be constructed to include the *ocs* enhancer element. This element was first identified as a 16 bp palindromic enhancer from the octopine synthase (*ocs*) gene of *Agrobacterium* (Ellis *et al.*, 1987), and is present in at least 10 other promoters (Bouchez *et al.*, 1989). It is proposed that the use of an enhancer element, such as the *ocs* element and particularly multiple copies of the element, will act to increase the level of transcription from adjacent promoters when applied in the context of plant transformation.

It is specifically envisioned that flavonoid and/or isoflavonoid biosynthesis coding sequences may be introduced under the control of novel promoters or enhancers, *etc.*, or homologous or tissue specific promoters or control elements. Vectors for use in tissue-specific targeting of genes in transgenic plants will typically include tissue-specific promoters and may also include other tissue-specific control elements such as enhancer sequences.

Promoters which direct specific or enhanced expression in certain plant tissues will be known to those of skill in the art in light of the present disclosure. These include, for example, the *rbcS* promoter, specific for green tissue; the *ocs*, *nos* and *mas* promoters which have higher activity in roots or wounded leaf tissue; a truncated (-90 to +8) 35S promoter which directs enhanced expression in roots, and an a-tubulin gene that also directs expression in roots.

B. Terminators

Transformation constructs prepared in accordance with the invention will typically include a 3' end DNA sequence that acts as a signal to terminate transcription and allow for the poly-adenylation of the mRNA produced by coding sequences operably linked to a flavonoid and/or isoflavonoid biosynthesis gene. In one embodiment of the invention, the native terminator of a flavonoid and/or isoflavonoid biosynthesis gene is used. Alternatively, a heterologous 3' end may enhance the expression of sense or antisense flavonoid and/or isoflavonoid biosynthesis genes. Terminators which are deemed to be particularly useful in this context include those from the nopaline synthase gene of *Agrobacterium tumefaciens* (nos 3' end) (Bevan *et al.,* 1983), the terminator for the T7 transcript from the octopine synthase gene of *Agrobacterium tumefaciens*, and the 3' end of the protease inhibitor I or II genes from potato or tomato. Regulatory elements such as an Adh intron (Callis *et al.,* 1987), sucrose synthase intron (Vasil *et al.,* 1989) or TMV omega element (Gallie *et al.,* 1989), may further be included where desired.

C. Transit or Signal Peptides

Sequences that are joined to the coding sequence of an expressed gene, which are removed post-translationally from the initial translation product and which facilitate the transport of the protein into or through intracellular or extracellular membranes, are termed transit (usually into vacuoles, vesicles, plastids and other intracellular organelles) and signal sequences (usually to the endoplasmic reticulum, golgi apparatus and outside of the cellular membrane). By facilitating the transport of the protein into compartments inside and outside the cell, these sequences may increase the accumulation of gene product protecting them from proteolytic degradation. These sequences also allow for additional mRNA sequences from

highly expressed genes to be attached to the coding sequence of the genes. Since mRNA being translated by ribosomes is more stable than naked mRNA, the presence of translatable mRNA in front of the gene may increase the overall stability of the mRNA transcript from the gene and thereby increase synthesis of the gene product. Since transit and signal sequences are usually post-translationally removed from the initial translation product, the use of these sequences allows for the addition of extra translated sequences that may not appear on the final polypeptide. It further is contemplated that targeting of certain proteins may be desirable in order to enhance the stability of the protein (U.S. Patent No. 5,545,818, incorporated herein by reference in its entirety).

Additionally, vectors may be constructed and employed in the intracellular targeting of a specific gene product within the cells of a transgenic plant or in directing a protein to the extracellular environment. This generally will be achieved by joining a DNA sequence encoding a transit or signal peptide sequence to the coding sequence of a particular gene. The resultant transit, or signal, peptide will transport the protein to a particular intracellular, or extracellular destination, respectively, and will then be post-translationally removed.

D. Marker Genes

By employing a selectable or screenable marker protein, one can provide or enhance the ability to identify transformants. "Marker genes" are genes that impart a distinct phenotype to cells expressing the marker protein and thus allow such transformed cells to be distinguished from cells that do not have the marker. Such genes may encode either a selectable or screenable marker, depending on whether the marker confers a trait which one can "select" for by chemical means, *i.e.*, through the use of a selective agent (*e.g.*, a herbicide, antibiotic, or the like), or whether it is simply a trait that one can identify through observation or testing, *i.e.*, by "screening" (*e.g.*, the green fluorescent protein). Of course, many examples of suitable marker proteins are known to the art and can be employed in the practice of the invention.

Included within the terms selectable or screenable markers also are genes which encode a "secretable marker" whose secretion can be detected as a means of identifying or

selecting for transformed cells. Examples include markers which are secretable antigens that can be identified by antibody interaction, or even secretable enzymes which can be detected by their catalytic activity. Secretable proteins fall into a number of classes, including small, diffusible proteins detectable, *e.g.*, by ELISA; small active enzymes detectable in extracellular solution (*e.g.*, α-amylase, β-lactamase, phosphinothricin acetyltransferase); and proteins that are inserted or trapped in the cell wall (*e.g.*, proteins that include a leader sequence such as that found in the expression unit of extensin or tobacco PR-S).

With regard to selectable secretable markers, the use of a gene that encodes a protein that becomes sequestered in the cell wall, and which protein includes a unique epitope is considered to be particularly advantageous. Such a secreted antigen marker would ideally employ an epitope sequence that would provide low background in plant tissue, a promoter-leader sequence that would impart efficient expression and targeting across the plasma membrane, and would produce protein that is bound in the cell wall and yet accessible to antibodies. A normally secreted wall protein modified to include a unique epitope would satisfy all such requirements.

Many selectable marker coding regions are known and could be used with the present invention including, but not limited to, *neo* (Potrykus *et al.,* 1985), which provides kanamycin resistance and can be selected for using kanamycin, G418, paromomycin, *etc.*; *bar*, which confers bialaphos or phosphinothricin resistance; a mutant EPSP synthase protein (Hinchee *et al.,* 1988) conferring glyphosate resistance; a nitrilase such as *bxn* from *Klebsiella ozaenae* which confers resistance to bromoxynil (Stalker *et al.,* 1988); a mutant acetolactate synthase (ALS) which confers resistance to imidazolinone, sulfonylurea or other ALS inhibiting chemicals (European Patent Application 154,204, 1985); a methotrexate resistant DHFR (Thillet *et al.,* 1988), a dalapon dehalogenase that confers resistance to the herbicide dalapon; or a mutated anthranilate synthase that confers resistance to 5-methyl tryptophan.

An illustrative embodiment of selectable marker capable of being used in systems to select transformants are those that encode the enzyme phosphinothricin acetyltransferase, such as the *bar* gene from *Streptomyces hygroscopicus* or the *pat* gene from *Streptomyces viridochromogenes*. The enzyme phosphinothricin acetyl transferase (PAT) inactivates the

active ingredient in the herbicide bialaphos, phosphinothricin (PPT). PPT inhibits glutamine synthetase (Murakami *et al.*, 1986; Twell *et al.*, 1989), causing rapid accumulation of ammonia and cell death.

Screenable markers that may be employed include a β-glucuronidase (GUS) or *uid*A gene which encodes an enzyme for which various chromogenic substrates are known; an R-locus gene, which encodes a product that regulates the production of anthocyanin pigments (red color) in plant tissues (Dellaporta *et al.*, 1988); a β-lactamase gene (Sutcliffe, 1978), which encodes an enzyme for which various chromogenic substrates are known (*e.g.*, PADAC, a chromogenic cephalosporin); a *xyl*E gene (Zukowsky *et al.*, 1983) which encodes a catechol dioxygenase that can convert chromogenic catechols; an α-amylase gene (Ikuta *et al.*, 1990); a tyrosinase gene (Katz *et al.*, 1983) which encodes an enzyme capable of oxidizing tyrosine to DOPA and dopaquinone which in turn condenses to form the easily-detectable compound melanin; a β-galactosidase gene, which encodes an enzyme for which there are chromogenic substrates; a luciferase (*lux*) gene (Ow *et al.*, 1986), which allows for bioluminescence detection; an aequorin gene (Prasher *et al.*, 1985) which may be employed in calcium-sensitive bioluminescence detection; or a gene encoding for green fluorescent protein (Sheen *et al.*, 1995; Haseloff *et al.*, 1997; Reichel *et al.*, 1996; Tian *et al.*, 1997; WO 97/41228).

Another screenable marker contemplated for use in the present invention is firefly luciferase, encoded by the *lux* gene. The presence of the *lux* gene in transformed cells may be detected using, for example, X-ray film, scintillation counting, fluorescent spectrophotometry, low-light video cameras, photon counting cameras or multiwell luminometry. It also is envisioned that this system may be developed for populational screening for bioluminescence, such as on tissue culture plates, or even for whole plant screening. The gene which encodes green fluorescent protein (GFP) is also contemplated as a particularly useful reporter gene (Sheen *et al.*, 1995; Haseloff *et al.*, 1997; Reichel *et al.*, 1996; Tian *et al.*, 1997; WO 97/41228). Expression of green fluorescent protein may be visualized in a cell or plant as fluorescence following illumination by particular wavelengths of light.

II. Antisense Constructs

Antisense treatments represent one way of altering flavonoid and/or isoflavonoid biosynthesis in accordance with the invention. In one embodiment of the invention, constructs comprising an antisense flavanone 3-hydroxylase sequence may be used to increase isoflavone production and accumulation in plants by decreasing or effectively eliminating flavanone 3-hydroxylase activity in a plant. Accordingly, this may be used to increase anthocyanin accumulation in a plant or given plant tissue. As such, antisense technology may be used to "knock-out" the function of a flavanone 3-hydroxylase gene or another flavonoid and/or isoflavonoid biosynthesis gene or homologous sequences thereof.

Antisense methodology takes advantage of the fact that nucleic acids tend to pair with "complementary" sequences. By complementary, it is meant that polynucleotides are those which are capable of base-pairing according to the standard Watson-Crick complementarity rules. That is, the larger purines will base pair with the smaller pyrimidines to form combinations of guanine paired with cytosine (G:C) and adenine paired with either thymine (A:T) in the case of DNA, or adenine paired with uracil (A:U) in the case of RNA. Inclusion of less common bases such as inosine, 5-methylcytosine, 6-methyladenine, hypoxanthine and others in hybridizing sequences does not interfere with pairing.

Targeting double-stranded (ds) DNA with polynucleotides leads to triple-helix formation; targeting RNA will lead to double-helix formation. Antisense polynucleotides, when introduced into a target cell, specifically bind to their target polynucleotide and interfere with transcription, RNA processing, transport, translation and/or stability. Antisense RNA constructs, or DNA encoding such antisense RNA's, may be employed to inhibit gene transcription or translation or both within a host cell, either in vitro or in vivo, such as within a host animal, including a human subject.

Antisense constructs may be designed to bind to the promoter and other control regions, exons, introns or even exon-intron boundaries of a gene. It is contemplated that the most effective antisense constructs will include regions complementary to intron/exon splice junctions. Thus, it is proposed that a preferred embodiment includes an antisense construct

with complementarity to regions within 50-200 bases of an intron-exon splice junction. It has been observed that some exon sequences can be included in the construct without seriously affecting the target selectivity thereof. The amount of exonic material included will vary depending on the particular exon and intron sequences used. One can readily test whether too much exon DNA is included simply by testing the constructs *in vitro* to determine whether normal cellular function is affected or whether the expression of related genes having complementary sequences is affected.

As stated above, "complementary" or "antisense" means polynucleotide sequences that are substantially complementary over their entire length and have very few base mismatches. For example, sequences of fifteen bases in length may be termed complementary when they have complementary nucleotides at thirteen or fourteen positions. Naturally, sequences which are completely complementary will be sequences which are entirely complementary throughout their entire length and have no base mismatches. Other sequences with lower degrees of homology also are contemplated. For example, an antisense construct which has limited regions of high homology, but also contains a non-homologous region (*e.g.*, ribozyme; see above) could be designed. These molecules, though having less than 50% homology, would bind to target sequences under appropriate conditions.

It may be advantageous to combine portions of genomic DNA with cDNA or synthetic sequences to generate specific constructs. For example, where an intron is desired in the ultimate construct, a genomic clone will need to be used. The cDNA or a synthesized polynucleotide may provide more convenient restriction sites for the remaining portion of the construct and, therefore, would be used for the rest of the sequence.

III. Tissue Cultures

Tissue cultures may be used in certain transformation techniques for the preparation of cells for transformation and for the regeneration of plants therefrom. Maintenance of tissue cultures requires use of media and controlled environments. "Media" refers to the numerous nutrient mixtures that are used to grow cells *in vitro*, that is, outside of the intact living organism. The medium usually is a suspension of various categories of ingredients (salts,

amino acids, growth regulators, sugars, buffers) that are required for growth of most cell types. However, each specific cell type requires a specific range of ingredient proportions for growth, and an even more specific range of formulas for optimum growth. Rate of cell growth also will vary among cultures initiated with the array of media that permit growth of that cell type.

Nutrient media is prepared as a liquid, but this may be solidified by adding the liquid to materials capable of providing a solid support. Agar is most commonly used for this purpose. Bactoagar, Hazelton agar, Gelrite, and Gelgro are specific types of solid support that are suitable for growth of plant cells in tissue culture.

Some cell types will grow and divide either in liquid suspension or on solid media. As disclosed herein, plant cells will grow in suspension or on solid medium, but regeneration of plants from suspension cultures typically requires transfer from liquid to solid media at some point in development. The type and extent of differentiation of cells in culture will be affected not only by the type of media used and by the environment, for example, pH, but also by whether media is solid or liquid.

Tissue that can be grown in a culture includes meristem cells, Type I, Type II, and Type III callus, immature embryos and gametic cells such as microspores, pollen, sperm and egg cells. Type I, Type II, and Type III callus may be initiated from tissue sources including, but not limited to, immature embryos, seedling apical meristems, root, leaf, microspores and the like. Those cells which are capable of proliferating as callus also are recipient cells for genetic transformation.

Somatic cells are of various types. Embryogenic cells are one example of somatic cells which may be induced to regenerate a plant through embryo formation. Non-embryogenic cells are those which typically will not respond in such a fashion. Certain techniques may be used that enrich recipient cells within a cell population. For example, Type II callus development, followed by manual selection and culture of friable, embryogenic tissue, generally results in an enrichment of cells. Manual selection techniques which can be employed to select target cells may include, *e.g.*, assessing cell morphology and

differentiation, or may use various physical or biological means. Cryopreservation also is a possible method of selecting for recipient cells.

Manual selection of recipient cells, *e.g.*, by selecting embryogenic cells from the surface of a Type II callus, is one means that may be used in an attempt to enrich for particular cells prior to culturing (whether cultured on solid media or in suspension).

Where employed, cultured cells may be grown either on solid supports or in the form of liquid suspensions. In either instance, nutrients may be provided to the cells in the form of media, and environmental conditions controlled. There are many types of tissue culture media comprised of various amino acids, salts, sugars, growth regulators and vitamins. Most of the media employed in the practice of the invention will have some similar components, but may differ in the composition and proportions of their ingredients depending on the particular application envisioned. For example, various cell types usually grow in more than one type of media, but will exhibit different growth rates and different morphologies, depending on the growth media. In some media, cells survive but do not divide. Various types of media suitable for culture of plant cells previously have been described. Examples of these media include, but are not limited to, the N6 medium described by Chu *et al.*,(1975) and MS media (Murashige and Skoog, 1962).

IV. Methods for Genetic Transformation

Suitable methods for transformation of plant or other cells for use with the current invention are believed to include virtually any method by which DNA can be introduced into a cell, such as by direct delivery of DNA such as by PEG-mediated transformation of protoplasts (Omirulleh *et al.,* 1993), by desiccation/inhibition-mediated DNA uptake (Potrykus *et al.,* 1985), by electroporation (U.S. Patent No. 5,384,253, specifically incorporated herein by reference in its entirety), by agitation with silicon carbide fibers (Kaeppler *et al.,* 1990; U.S. Patent No. 5,302,523, specifically incorporated herein by reference in its entirety; and U.S. Patent No. 5,464,765, specifically incorporated herein by reference in its entirety), by *Agrobacterium*-mediated transformation (U.S. Patent No. 5,591,616 and U.S. Patent No. 5,563,055; both specifically incorporated herein by reference)

and by acceleration of DNA coated particles (U.S. Patent No. 5,550,318; U.S. Patent No. 5,538,877; and U.S. Patent No. 5,538,880; each specifically incorporated herein by reference in its entirety), *etc.* Through the application of techniques such as these, the cells of virtually any plant species may be stably transformed, and these cells developed into transgenic plants.

A. *Agrobacterium*-mediated Transformation

Agrobacterium-mediated transfer is a widely applicable system for introducing genes into plant cells because the DNA can be introduced into whole plant tissues, thereby bypassing the need for regeneration of an intact plant from a protoplast. The use of *Agrobacterium*-mediated plant integrating vectors to introduce DNA into plant cells is well known in the art. See, for example, the methods described by Fraley *et al.,* (1985), Rogers *et al.,* (1987) and U.S. Patent No. 5,563,055, specifically incorporated herein by reference in its entirety.

Agrobacterium-mediated transformation is most efficient in dicotyledonous plants and is the preferable method for transformation of dicots, including *Arabidopsis*, tobacco, tomato, alfalfa and potato. Indeed, while *Agrobacterium*-mediated transformation has been routinely used with dicotyledonous plants for a number of years, it has only recently become applicable to monocotyledonous plants. Advances in *Agrobacterium*-mediated transformation techniques have now made the technique applicable to nearly all monocotyledonous plants. For example, *Agrobacterium*-mediated transformation techniques have now been applied to rice (Hiei *et al.,* 1997; U.S. Patent No. 5,591,616, specifically incorporated herein by reference in its entirety), wheat (McCormac *et al.,* 1998), barley (Tingay *et al.,* 1997; McCormac *et al.,* 1998), alfalfa (Thomas *et al.,* 1990) and maize (Ishidia *et al.,* 1996).

Modern *Agrobacterium* transformation vectors are capable of replication in *E. coli* as well as *Agrobacterium*, allowing for convenient manipulations as described (Klee *et al.,* 1985). Moreover, recent technological advances in vectors for *Agrobacterium*-mediated gene transfer have improved the arrangement of genes and restriction sites in the vectors to facilitate the construction of vectors capable of expressing various polypeptide coding genes. The vectors described (Rogers *et al.,* 1987) have convenient multi-linker regions flanked by a

promoter and a polyadenylation site for direct expression of inserted polypeptide coding genes and are suitable for present purposes. In addition, *Agrobacterium* containing both armed and disarmed Ti genes can be used for the transformations. In those plant strains where *Agrobacterium*-mediated transformation is efficient, it is the method of choice because of the facile and defined nature of the gene transfer.

B. Electroporation

To effect transformation by electroporation, one may employ either friable tissues, such as a suspension culture of cells or embryogenic callus or alternatively one may transform immature embryos or other organized tissue directly. In this technique, one would partially degrade the cell walls of the chosen cells by exposing them to pectin-degrading enzymes (pectolyases) or mechanically wounding in a controlled manner. Examples of some species which have been transformed by electroporation of intact cells include maize (U.S. Patent No. 5,384,253; Rhodes *et al.,* 1995; D'Halluin *et al.,* 1992), wheat (Zhou *et al.,* 1993), tomato (Hou and Lin, 1996), soybean (Christou *et al.,* 1987) and tobacco (Lee *et al.,* 1989).

One also may employ protoplasts for electroporation transformation of plants (Bates, 1994; Lazzeri, 1995). For example, the generation of transgenic soybean plants by electroporation of cotyledon-derived protoplasts is described by Dhir and Widholm in Intl. Patent Appl. Publ. No. WO 9217598 (specifically incorporated herein by reference). Other examples of species for which protoplast transformation has been described include barley (Lazerri, 1995), sorghum (Battraw *et al.,* 1991), maize (Bhattacharjee *et al.,* 1997), wheat (He *et al.,* 1994) and tomato (Tsukada, 1989).

C. Microprojectile Bombardment

Another method for delivering transforming DNA segments to plant cells in accordance with the invention is microprojectile bombardment (U.S. Patent No. 5,550,318; U.S. Patent No. 5,538,880; U.S. Patent No. 5,610,042; and PCT Application WO 94/09699; each of which is specifically incorporated herein by reference in its entirety). In this method, particles may be coated with nucleic acids and delivered into cells by a propelling force. Exemplary particles include those comprised of tungsten, platinum, and preferably, gold. It is

contemplated that in some instances DNA precipitation onto metal particles would not be necessary for DNA delivery to a recipient cell using microprojectile bombardment. However, it is contemplated that particles may contain DNA rather than be coated with DNA. Hence, it is proposed that DNA-coated particles may increase the level of DNA delivery via particle bombardment but are not, in and of themselves, necessary.

For the bombardment, cells in suspension are concentrated on filters or solid culture medium. Alternatively, immature embryos or other target cells may be arranged on solid culture medium. The cells to be bombarded are positioned at an appropriate distance below the macroprojectile stopping plate.

An illustrative embodiment of a method for delivering DNA into plant cells by acceleration is the Biolistics Particle Delivery System, which can be used to propel particles coated with DNA or cells through a screen, such as a stainless steel or Nytex screen, onto a filter surface covered with monocot plant cells cultured in suspension. The screen disperses the particles so that they are not delivered to the recipient cells in large aggregates. Microprojectile bombardment techniques are widely applicable, and may be used to transform virtually any plant species. Examples of species for which have been transformed by microprojectile bombardment include monocot species such as maize (PCT Application WO 95/06128), barley (Ritala *et al.,* 1994; Hensgens *et al.,* 1993), wheat (U.S. Patent No. 5,563,055, specifically incorporated herein by reference in its entirety), rice (Hensgens *et al.,* 1993), oat (Torbet *et al.,* 1995; Torbet *et al.,* 1998), rye (Hensgens *et al.,* 1993), sugarcane (Bower *et al.,* 1992), and sorghum (Casa *et al.,* 1993; Hagio *et al.,* 1991); as well as a number of dicots including tobacco (Tomes *et al.,* 1990; Buising and Benbow, 1994), soybean (U.S. Patent No. 5,322,783, specifically incorporated herein by reference in its entirety), sunflower (Knittel *et al.,* 1994), peanut (Singsit *et al.,* 1997), cotton (McCabe and Martinell, 1993), tomato (VanEck *et al.,* 1995), and legumes in general (U.S. Patent No. 5,563,055, specifically incorporated herein by reference in its entirety).

D. Other Transformation Methods

Transformation of protoplasts can be achieved using methods based on calcium phosphate precipitation, polyethylene glycol treatment, electroporation, and combinations of these treatments (see, *e.g.*, Potrykus *et al.*, 1985; Lorz *et al.*, 1985; Omirulleh *et al.*, 1993; Fromm *et al.*, 1986; Uchimiya *et al.*, 1986; Callis *et al.*, 1987; Marcotte *et al.*, 1988).

Application of these systems to different plant strains depends upon the ability to regenerate that particular plant strain from protoplasts. Illustrative methods for the regeneration of cereals from protoplasts have been described (Toriyama *et al.*, 1986; Yamada *et al.*, 1986; Abdullah *et al.*, 1986; Omirulleh *et al.*, 1993 and U.S. Patent No. 5,508,184; each specifically incorporated herein by reference in its entirety). Examples of the use of direct uptake transformation of cereal protoplasts include transformation of rice (Ghosh-Biswas *et al.*, 1994), sorghum (Battraw and Hall, 1991), barley (Lazerri, 1995), oat (Zheng and Edwards, 1990) and maize (Omirulleh *et al.*, 1993).

To transform plant strains that cannot be successfully regenerated from protoplasts, other ways to introduce DNA into intact cells or tissues can be utilized. For example, regeneration of cereals from immature embryos or explants can be effected as described (Vasil, 1989). Also, silicon carbide fiber-mediated transformation may be used with or without protoplasting (Kaeppler, 1990; Kaeppler *et al.*, 1992; U.S. Patent No. 5,563,055, specifically incorporated herein by reference in its entirety). Transformation with this technique is accomplished by agitating silicon carbide fibers together with cells in a DNA solution. DNA passively enters as the cells are punctured. This technique has been used successfully with, for example, the monocot cereals maize (PCT Application WO 95/06128, specifically incorporated herein by reference in its entirety; (Thompson, 1995) and rice (Nagatani, 1997).

V. Production and Characterization of Stably Transformed Plants

After effecting delivery of exogenous DNA to recipient cells, the next steps generally concern identifying the transformed cells for further culturing and plant regeneration. In order to improve the ability to identify transformants, one may desire to employ a selectable

or screenable marker gene with a transformation vector prepared in accordance with the invention. In this case, one would then generally assay the potentially transformed cell population by exposing the cells to a selective agent or agents, or one would screen the cells for the desired marker gene trait.

A. Selection

It is believed that DNA is introduced into only a small percentage of target cells in any one experiment. In order to provide an efficient system for identification of those cells receiving DNA and integrating it into their genomes one may employ a means for selecting those cells that are stably transformed. One exemplary embodiment of such a method is to introduce into the host cell, a marker gene which confers resistance to some normally inhibitory agent, such as an antibiotic or herbicide. Examples of antibiotics which may be used include the aminoglycoside antibiotics neomycin, kanamycin and paromomycin, or the antibiotic hygromycin. Resistance to the aminoglycoside antibiotics is conferred by aminoglycoside phosphostransferase enzymes such as neomycin phosphotransferase II (NPT II) or NPT I, whereas resistance to hygromycin is conferred by hygromycin phosphotransferase.

Potentially transformed cells then are exposed to the selective agent. In the population of surviving cells will be those cells where, generally, the resistance-conferring gene has been integrated and expressed at sufficient levels to permit cell survival. Cells may be tested further to confirm stable integration of the exogenous DNA.

One herbicide which constitutes a desirable selection agent is the broad spectrum herbicide bialaphos. Bialaphos is a tripeptide antibiotic produced by *Streptomyces hygroscopicus* and is composed of phosphinothricin (PPT), an analogue of L-glutamic acid, and two L-alanine residues. Upon removal of the L-alanine residues by intracellular peptidases, the PPT is released and is a potent inhibitor of glutamine synthetase (GS), a pivotal enzyme involved in ammonia assimilation and nitrogen metabolism (Ogawa *et al.*, 1973). Synthetic PPT, the active ingredient in the herbicide Liberty™ also is effective as a

selection agent. Inhibition of GS in plants by PPT causes the rapid accumulation of ammonia and death of the plant cells.

The organism producing bialaphos and other species of the genus *Streptomyces* also synthesizes an enzyme phosphinothricin acetyl transferase (PAT) which is encoded by the *bar* gene in *Streptomyces hygroscopicus* and the *pat* gene in *Streptomyces viridochromogenes*. The use of the herbicide resistance gene encoding phosphinothricin acetyl transferase (PAT) is referred to in DE 3642 829 A, wherein the gene is isolated from *Streptomyces viridochromogenes*. In the bacterial source organism, this enzyme acetylates the free amino group of PPT preventing auto-toxicity (Thompson *et al.*, 1987). The *bar* gene has been cloned (Murakami *et al.*, 1986; Thompson *et al.*, 1987) and expressed in transgenic tobacco, tomato, potato (De Block *et al.*, 1987) *Brassica* (De Block *et al.*, 1989) and maize (U.S. Patent No. 5,550,318). In previous reports, some transgenic plants which expressed the resistance gene were completely resistant to commercial formulations of PPT and bialaphos in greenhouses.

Another example of a herbicide which is useful for selection of transformed cell lines in the practice of the invention is the broad spectrum herbicide glyphosate. Glyphosate inhibits the action of the enzyme EPSPS which is active in the aromatic amino acid biosynthetic pathway. Inhibition of this enzyme leads to starvation for the amino acids phenylalanine, tyrosine, and tryptophan and secondary metabolites derived thereof. U.S. Patent No. 4,535,060 describes the isolation of EPSPS mutations which confer glyphosate resistance on the *Salmonella typhimurium* gene for EPSPS, aroA. The EPSPS gene was cloned from *Zea mays* and mutations similar to those found in a glyphosate resistant aroA gene were introduced *in vitro*. Mutant genes encoding glyphosate resistant EPSPS enzymes are described in, for example, International Patent WO 97/4103. The best characterized mutant EPSPS gene conferring glyphosate resistance comprises amino acid changes at residues 102 and 106, although it is anticipated that other mutations will also be useful (PCT/WO97/4103).

To use the *bar*-bialaphos or the EPSPS-glyphosate selective system, transformed tissue is cultured for 0 - 28 days on nonselective medium and subsequently transferred to

medium containing from 1-3 mg/l bialaphos or 1-3 mM glyphosate as appropriate. While ranges of 1-3 mg/l bialaphos or 1-3 mM glyphosate will typically be preferred, it is proposed that ranges of 0.1-50 mg/l bialaphos or 0.1-50 mM glyphosate will find utility.

It further is contemplated that the herbicide DALAPON, 2,2-dichloropropionic acid, may be useful for identification of transformed cells. The enzyme 2,2-dichloropropionic acid dehalogenase (*deh*) inactivates the herbicidal activity of 2,2-dichloropropionic acid and therefore confers herbicidal resistance on cells or plants expressing a gene encoding the dehalogenase enzyme (Buchanan-Wollaston *et al.*, 1992; U.S. Patent No. 5,508,468; and U.S. Patent No. 5,508,468; each of the disclosures of which is specifically incorporated herein by reference in its entirety).

Alternatively, a gene encoding anthranilate synthase, which confers resistance to certain amino acid analogs, *e.g.*, 5-methyltryptophan or 6-methyl anthranilate, may be useful as a selectable marker gene. The use of an anthranilate synthase gene as a selectable marker was described in U.S. Patent No. 5,508,468.

An example of a screenable marker trait is the enzyme luciferase. In the presence of the substrate luciferin, cells expressing luciferase emit light which can be detected on photographic or x-ray film, in a luminometer (or liquid scintillation counter), by devices that enhance night vision, or by a highly light sensitive video camera, such as a photon counting camera. These assays are nondestructive and transformed cells may be cultured further following identification. The photon counting camera is especially valuable as it allows one to identify specific cells or groups of cells which are expressing luciferase and manipulate those in real time. Another screenable marker which may be used in a similar fashion is the gene coding for green fluorescent protein.

It further is contemplated that combinations of screenable and selectable markers will be useful for identification of transformed cells. In some cell or tissue types a selection agent, such as bialaphos or glyphosate, may either not provide enough killing activity to clearly recognize transformed cells or may cause substantial nonselective inhibition of transformants and nontransformants alike, thus causing the selection technique to not be effective. It is

proposed that selection with a growth inhibiting compound, such as bialaphos or glyphosate at concentrations below those that cause 100% inhibition followed by screening of growing tissue for expression of a screenable marker gene such as luciferase would allow one to recover transformants from cell or tissue types that are not amenable to selection alone. It is proposed that combinations of selection and screening may enable one to identify transformants in a wider variety of cell and tissue types. This may be efficiently achieved using a gene fusion between a selectable marker gene and a screenable marker gene, for example, between an NPTII gene and a GFP gene.

B. Regeneration and Seed Production

Cells that survive the exposure to the selective agent, or cells that have been scored positive in a screening assay, may be cultured in media that supports regeneration of plants. In an exemplary embodiment, MS and N6 media may be modified by including further substances such as growth regulators. One such growth regulator is dicamba or 2,4-D. However, other growth regulators may be employed, including NAA, NAA + 2,4-D or picloram. Media improvement in these and like ways has been found to facilitate the growth of cells at specific developmental stages. Tissue may be maintained on a basic media with growth regulators until sufficient tissue is available to begin plant regeneration efforts, or following repeated rounds of manual selection, until the morphology of the tissue is suitable for regeneration, at least 2 wk, then transferred to media conducive to maturation of embryoids. Cultures are transferred every 2 wk on this medium. Shoot development will signal the time to transfer to medium lacking growth regulators.

The transformed cells, identified by selection or screening and cultured in an appropriate medium that supports regeneration, will then be allowed to mature into plants. Developing plantlets are transferred to soiless plant growth mix, and hardened, *e.g.*, in an environmentally controlled chamber, for example, at about 85% relative humidity, 600 ppm CO_2, and 25-250 microeinsteins m^{-2} s^{-1} of light. Plants are preferably matured either in a growth chamber or greenhouse. Plants can be regenerated from about 6 wk to 10 months after a transformant is identified, depending on the initial tissue. During regeneration, cells are grown on solid media in tissue culture vessels. Illustrative embodiments of such vessels are

petri dishes and Plant Cons. Regenerating plants are preferably grown at about 19 to 28°C. After the regenerating plants have reached the stage of shoot and root development, they may be transferred to a greenhouse for further growth and testing.

Seeds on transformed plants may occasionally require embryo rescue due to cessation of seed development and premature senescence of plants. To rescue developing embryos, they are excised from surface-disinfected seeds 10-20 days post-pollination and cultured. An embodiment of media used for culture at this stage comprises MS salts, 2% sucrose, and 5.5 g/l agarose. In embryo rescue, large embryos (defined as greater than 3 mm in length) are germinated directly on an appropriate media. Embryos smaller than that may be cultured for 1 wk on media containing the above ingredients along with 10^{-5}M abscisic acid and then transferred to growth regulator-free medium for germination.

C. Characterization

To confirm the presence of the exogenous DNA or "transgene(s)" in the regenerating plants, a variety of assays may be performed. Such assays include, for example, "molecular biological" assays, such as Southern and Northern blotting and PCR™; "biochemical" assays, such as detecting the presence of a protein product, *e.g.*, by immunological means (ELISAs and Western blots) or by enzymatic function; plant part assays, such as leaf or root assays; and also, by analyzing the phenotype of the whole regenerated plant.

D. DNA Integration, RNA Expression and Inheritance

Genomic DNA may be isolated from cell lines or any plant parts to determine the presence of the exogenous gene through the use of techniques well known to those skilled in the art. Note, that intact sequences will not always be present, presumably due to rearrangement or deletion of sequences in the cell. The presence of DNA elements introduced through the methods of this invention may be determined, for example, by polymerase chain reaction (PCR™). Using this technique, discreet fragments of DNA are amplified and detected by gel electrophoresis. This type of analysis permits one to determine whether a gene is present in a stable transformant, but does not prove integration of the introduced gene

into the host cell genome. It is typically the case, however, that DNA has been integrated into the genome of all transformants that demonstrate the presence of the gene through PCR™ analysis. In addition, it is not typically possible using PCR™ techniques to determine whether transformants have exogenous genes introduced into different sites in the genome, *i.e.*, whether transformants are of independent origin. It is contemplated that using PCR™ techniques it would be possible to clone fragments of the host genomic DNA adjacent to an introduced gene.

Positive proof of DNA integration into the host genome and the independent identities of transformants may be determined using the technique of Southern hybridization. Using this technique specific DNA sequences that were introduced into the host genome and flanking host DNA sequences can be identified. Hence the Southern hybridization pattern of a given transformant serves as an identifying characteristic of that transformant. In addition it is possible through Southern hybridization to demonstrate the presence of introduced genes in high molecular weight DNA, *i.e.*, confirm that the introduced gene has been integrated into the host cell genome. The technique of Southern hybridization provides information that is obtained using PCR™, *e.g.*, the presence of a gene, but also demonstrates integration into the genome and characterizes each individual transformant.

It is contemplated that using the techniques of dot or slot blot hybridization which are modifications of Southern hybridization techniques one could obtain the same information that is derived from PCR™, *e.g.*, the presence of a gene.

Both PCR™ and Southern hybridization techniques can be used to demonstrate transmission of a transgene to progeny. In most instances the characteristic Southern hybridization pattern for a given transformant will segregate in progeny as one or more Mendelian genes (Spencer *et al.*, 1992) indicating stable inheritance of the transgene.

Whereas DNA analysis techniques may be conducted using DNA isolated from any part of a plant, RNA will only be expressed in particular cells or tissue types and hence it will be necessary to prepare RNA for analysis from these tissues. PCR™ techniques also may be used for detection and quantitation of RNA produced from introduced genes. In this

application of PCR™ it is first necessary to reverse transcribe RNA into DNA, using enzymes such as reverse transcriptase, and then through the use of conventional PCR™ techniques amplify the DNA. In most instances PCR™ techniques, while useful, will not demonstrate integrity of the RNA product. Further information about the nature of the RNA product may be obtained by Northern blotting. This technique will demonstrate the presence of an RNA species and give information about the integrity of that RNA. The presence or absence of an RNA species also can be determined using dot or slot blot Northern hybridizations. These techniques are modifications of Northern blotting and will only demonstrate the presence or absence of an RNA species.

E. Gene Expression

While Southern blotting and PCR™ may be used to detect the gene(s) in question, they do not provide information as to whether the corresponding protein is being expressed. Expression may be evaluated by specifically identifying the protein products of the introduced genes or evaluating the phenotypic changes brought about by their expression.

Assays for the production and identification of specific proteins may make use of physical-chemical, structural, functional, or other properties of the proteins. Unique physical-chemical or structural properties allow the proteins to be separated and identified by electrophoretic procedures, such as native or denaturing gel electrophoresis or isoelectric focusing, or by chromatographic techniques such as ion exchange or gel exclusion chromatography. The unique structures of individual proteins offer opportunities for use of specific antibodies to detect their presence in formats such as an ELISA assay. Combinations of approaches may be employed with even greater specificity such as western blotting in which antibodies are used to locate individual gene products that have been separated by electrophoretic techniques. Additional techniques may be employed to absolutely confirm the identity of the product of interest such as evaluation by amino acid sequencing following purification. Although these are among the most commonly employed, other procedures may be additionally used.

Assay procedures also may be used to identify the expression of proteins by their functionality, especially the ability of enzymes to catalyze specific chemical reactions involving specific substrates and products. These reactions may be followed by providing and quantifying the loss of substrates or the generation of products of the reactions by physical or chemical procedures. Examples are as varied as the enzyme to be analyzed and may include assays for PAT enzymatic activity by following production of radiolabeled acetylated phosphinothricin from phosphinothricin and ^{14}C-acetyl CoA or for anthranilate synthase activity by following loss of fluorescence of anthranilate, to name two.

Very frequently the expression of a gene product is determined by evaluating the phenotypic results of its expression. These assays also may take many forms including but not limited to analyzing changes in the chemical composition, morphology, or physiological properties of the plant. Chemical composition may be altered by expression of genes encoding enzymes or storage proteins which change amino acid composition and may be detected by amino acid analysis, or by enzymes which change starch quantity which may be analyzed by near infrared reflectance spectrometry. Morphological changes may include greater stature or thicker stalks. Most often changes in response of plants or plant parts to imposed treatments are evaluated under carefully controlled conditions termed bioassays.

VI. Breeding Plants of the Invention

In addition to direct transformation of a particular plant genotype with a construct prepared according to the current invention, transgenic plants may be made by crossing a plant having a selected DNA of the invention to a second plant lacking the construct. Plant breeding techniques may also be used to introduce a transgenic or non-transgenic mutated or defective flavanone 3-hydroxylase into a plant. In this manner, flavanone 3-hydroxylase can be effectively down regulated. By creating plants homozygous for a mutant allele, flavanone 3-hydroxylase activity can be eliminated in the plant.

As set forth above, a selected flavonoid and/or isoflavonoid biosynthesis gene can be introduced into a particular plant variety by crossing, without the need for ever directly transforming a plant of that given variety. Therefore, the current invention not only

encompasses a plant directly transformed or regenerated from cells which have been transformed in accordance with the current invention, but also the progeny of such plants. As used herein the term "progeny" denotes the offspring of any generation of a parent plant prepared in accordance with the instant invention, wherein the progeny comprises a selected DNA construct prepared in accordance with the invention. "Crossing" a plant to provide a plant line having one or more added transgenes or alleles relative to a starting plant line, as disclosed herein, is defined as the techniques that result in a particular sequence being introduced into a plant line by crossing a starting line with a donor plant line that comprises a transgene or allele of the invention. To achieve this one could, for example, perform the following steps:

(a) plant seeds of the first (starting line) and second (donor plant line that comprises a desired transgene or allele) parent plants;

(b) grow the seeds of the first and second parent plants into plants that bear flowers;

(c) pollinate a flower from the first parent plant with pollen from the second parent plant; and

(d) harvest seeds produced on the parent plant bearing the fertilized flower.

Backcrossing is herein defined as the process including the steps of:

(a) crossing a plant of a first genotype containing a desired gene, DNA sequence or element to a plant of a second genotype lacking said desired gene, DNA sequence or element;

(b) selecting one or more progeny plant containing the desired gene, DNA sequence or element;

(c) crossing the progeny plant to a plant of the second genotype; and

(d) repeating steps (b) and (c) for the purpose of transferring a desired DNA sequence from a plant of a first genotype to a plant of a second genotype.

Introgression of a DNA element into a plant genotype is defined as the result of the process of backcross conversion. A plant genotype into which a DNA sequence has been introgressed may be referred to as a backcross converted genotype, line, inbred, or hybrid. Similarly a plant genotype lacking the desired DNA sequence may be referred to as an unconverted genotype, line, inbred, or hybrid.

VII. Nutraceuticals

Nutraceutical compositions are preparations of natural ingredients that are multi-component systems consisting of preferably synergistic natural products and supplements to promote good health. The plants provided by the invention contain increased isoflavonoid content, which has certain health benefits, and thus these plants may be used for the preparation of nutraceutical compositions. Nutraceutical compositions can be derived from plant tissue. Information about numerous plants and herbs that have been used to prepare nutraceutical compositions has been compiled and is available in publications including the *German Commission E Monographs*, *Botanical Safety Handbook*, and *HerbalGram,* a quarterly publication of the American Botanical Council which references numerous clinical trials that have been performed using nutraceuticals.

Information on description and constituents, modern uses, dosage (in a variety of forms), actions, contraindications, side effects, interactions with conventional drugs, mode of administration, duration of application, regulatory status, AHPA botanical safety rating, and comments are available for a number of plants and include among others bilberry, cascara, cat's claw, cayenne, cranberry, devil's claw, dong quai, echinacea, evening primrose oil, feverfew, garlic, ginger, ginkgo, Asian ginseng, Siberian ginseng, goldenseal, gotu kola, grape seed, green tea, hawthorn, kava, licorice, milk thistle, saw palmetto, St. John's wort, and valerian.

The actions of these nutraceutical compounds may be fast or/and short-term or may help achieve long-term health objectives. Nutraceutical compositions may comprise dried and ground plant tissue or extracts from these tissues in a pharmacologically acceptable medium as a natural approach for treatment of various ailments. The nutraceutical compositions may be contained in a medium such as a buffer, a solvent, a diluent, an inert carrier, an oil, a creme, or an edible material. The nutraceutical may be orally administered and may be in the form of a tablet or a capsule. Alternatively the nutraceutical may be in the form of an ointment which has extracts of plant tissue in an oil or cream which can be topically applied to the skin.

VIII. Definitions

Isoflavone biosynthesis gene: A gene encoding a polypeptide that catalyzes one or more steps in the plant biosynthesis of isoflavones.

Flavanone biosynthesis gene: A gene encoding a polypeptide that catalyzes one or more steps in the plant biosynthesis of flavanones.

Expression: The combination of intracellular processes, including transcription and translation undergone by a coding DNA molecule such as a structural gene to produce a polypeptide.

Genetic Transformation: A process of introducing a DNA sequence or construct (*e.g.*, a vector or expression cassette) into a cell or protoplast in which that exogenous DNA is incorporated into a chromosome or is capable of autonomous replication.

Heterologous: A sequence which is not normally present in a given host genome in the genetic context in which the sequence is currently found In this respect, the sequence may be native to the host genome, but be rearranged with respect to other genetic sequences within the host sequence. For example, a regulatory sequence may be heterologous in that it is linked to a different coding sequence relative to the native regulatory sequence.

Obtaining: When used in conjunction with a transgenic plant cell or transgenic plant, obtaining means either transforming a non-transgenic plant cell or plant to create the transgenic plant cell or plant, or planting transgenic plant seed to produce the transgenic plant cell or plant. Such a transgenic plant seed may be from an R_0 transgenic plant or may be from a progeny of any generation thereof that inherits a given transgenic sequence from a starting transgenic parent plant.

Promoter: A recognition site on a DNA sequence or group of DNA sequences that provides an expression control element for a structural gene and to which RNA polymerase specifically binds and initiates RNA synthesis (transcription) of that gene.

R_0 transgenic plant: A plant that has been genetically transformed or has been regenerated from a plant cell or cells that have been genetically transformed.

Regeneration: The process of growing a plant from a plant cell (*e.g.*, plant protoplast, callus or explant).

Selected DNA: A DNA segment which one desires to introduce or has introduced into a plant genome by genetic transformation.

Transformation construct: A chimeric DNA molecule which is designed for introduction into a host genome by genetic transformation. Preferred transformation constructs will comprise all of the genetic elements necessary to direct the expression of one or more exogenous genes. In particular embodiments of the instant invention, it may be desirable to introduce a transformation construct into a host cell in the form of an expression cassette.

Transformed cell: A cell the DNA complement of which has been altered by the introduction of an exogenous DNA molecule into that cell.

Transgene: A segment of DNA which has been incorporated into a host genome or is capable of autonomous replication in a host cell and is capable of causing the expression of one or more mRNA(s), which may or may not be coding sequences. Exemplary transgenes

will provide the host cell, or plants regenerated therefrom, with a novel phenotype relative to the corresponding non-transformed cell or plant. Transgenes may be directly introduced into a plant by genetic transformation, or may be inherited from a plant of any previous generation which was transformed with the DNA segment.

Transgenic plant: A plant or progeny plant of any subsequent generation derived therefrom, wherein the DNA of the plant or progeny thereof contains an introduced exogenous DNA segment not naturally present in a non-transgenic plant of the same strain. The transgenic plant may additionally contain sequences which are native to the plant being transformed, but wherein the "exogenous" gene has been altered in order to alter the level or pattern of expression of the gene, for example, by use of one or more heterologous regulatory or other elements.

Vector: A DNA molecule capable of replication in a host cell and/or to which another DNA segment can be operatively linked so as to bring about replication of the attached segment. A plasmid is an exemplary vector.

IX. Examples

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

Example 1

Expression of soybean CYP93C1v2 in *Arabidopsis thaliana*

The open reading frame of the soybean CYP93C1v2 IFS cDNA (Genbank Accession #AFO22462; SEQ ID NO:1; Steele *et al.,* 1999) was cloned into the binary vector pCHF3, under control of the constitutive cauliflower mosaic virus 35S promoter, as described in PCT/US00/05915. Seedlings of *Arabidopsis thaliana* ecotype Columbia were transformed using a floral vacuum infiltration method (Clough and Bent, 1998) and selected on Petri plates with the antibiotic kanamycin. Resistant seedlings were self pollinated, and resultant T_2 progeny subjected to molecular (DNA and RNA gel blot) and phytochemical analysis by standard methodology to verify transformation.

Leaves of *Arabidopsis* plant 15B expressing CYP93C1v2 were extracted in acetone/methanol and analyzed for flavonoid content by HPLC, with effluent monitored by diode array detection. Untransformed *Arabidopsis* leaves contained a series of glycosides of the flavonols kaempferol and quercetin (Graham, 1998), and the identity of these compounds was confirmed by their UV absorption characteristics and shifts in peak positions following pre-treatment of extracts with almond ß-glucosidase. The compounds were shown to correspond to the flavonol rhamnosides and glucorhamnosides previously reported (Graham, 1998). In contrast, corresponding extracts from leaves of *Arabidopsis* expressing CYP93C1v2 contained three extra peaks. All three peaks exhibited UV absorption spectra similar to that of authentic genistein. Following treatment with ß-glucosidase, one compound disappeared, and a new peak appeared that co-chromatographed with a sample of authentic genistein, indicating that this compound is a ß-glucoside of genistein. The other two new compounds were conjugates in which a sugar other than glucose is directly linked to the isoflavone.

LC/MS was used for further identification of the genistein conjugates found in the transgenic *Arabidopsis*. The results identified the compounds as glucose-genistein, glucose-rhamnose-genistein, and rhamnose-genistein. Although MS analysis clearly identified the molecular weights of the sugars conjugated to the genistein, it did not reveal the position of

attachment, which could be at position 7 (the most likely, based on the common structures of other flavonoid and isoflavonoid glycosides), 5, or 4'. Isoflavones with a free 7-hydroxyl group exhibit bathochromic shifts following ionization of this hydroxyl group in the presence of sodium acetate (Mabry, 1970). In the case of genistein, there is a 10 nm shift of the absorption maximum to a higher wavelength, whereas genistin (7-*O*-glucosyl genistein) does not exhibit such a shift. No shifts in absorption maxima were observed for the genistein conjugates following treatment with sodium acetate, confirming the expected linkage of the glycans to the 7-position.

Feeding isoflavones to plant cell cultures can result in their incorporation into the insoluble cell wall fraction (Park, 1995). To determine whether *Arabidopsis* could similarly metabolize the introduced foreign isoflavone genistein, cell walls were prepared, the phenolic fraction was liberated by alkaline hydrolysis, and the components were analyzed by HPLC. The analyses revealed several wall-bound hydroxycinnamic acids, including ferulic acid, to be present in equal amounts in empty vector and CYP93C1v2 transformants. However, there was no evidence for the presence of genistein in the cell wall fraction from the CYP93C1v2 transformants.

Free isoflavones rarely accumulate constitutively in plants; they are usually present as glucosides and malonyl glucosides in legumes (Graham, 1990; Park *et al.*, 1992; Sumner *et al.*, 1996). Likewise, *Arabidopsis* glycosylates the introduced genistein, presumably using the same glycosyl transferases that are involved in conjugation of the plant's natural flavonoid end products, the flavonols kaempferol and quercetin. Studies on uptake of dietary genistein have suggested that the free aglycone is highly bioavailable (Sfakianos *et al.*, 1997), and isoflavone glycosides are hydrolyzed to the aglycones by lactobacilli, *Bacteroides* and *Bifidobacteria* in the intestinal flora. Glycosylation is, therefore, a favorable trait for engineered nutraceutical products, as it results in their storage in the vacuole, away from further potential metabolism, in a form that does not compromise bioavailability.

Example 2

Expression of alfalfa CHI in *Arabidopsis thaliana*

The open reading frame of the alfalfa chalcone isomerase MSCHI1 cDNA (Genbank Accession # M91079; SEQ ID NO:3; McKhann and Hirsch, 1994) was cloned into the binary vector pCHF3, under control of the constitutive cauliflower mosaic virus 35S promoter. Seedlings of *Arabidopsis thaliana* ecotype Columbia were transformed using a floral vacuum infiltration method (Clough and Bent, 1998), and selected on Petri plates with the antibiotic kanamycin. Resistant seedlings were self pollinated, and resultant T_2 progeny subjected to molecular and phytochemical analysis by methods known in the art to verify transformation.

CHI transformants were analyzed for alfalfa CHI transcript levels by RNA gel blot analysis. Six independent lines had very high expression, and four further lines exhibited low to moderate expression. The highest expressing line, named 4-11, and two other strongly expressing lines, 4-9 and 4-25, were used for further analysis. Leaves of the three *Arabidopsis* lines expressing alfalfa CHI were sequentially extracted in acetone and acetone/methanol (1:1) and analyzed for flavonoid content by HPLC, with effluent monitored by diode array detection. FIG. 2A shows HPLC traces of non-hydrolyzed leaf extracts from a control line, showing the presence of a number of rhamnose and/or glucose conjugates of kaempferol and quercetin. FIG. 2B shows a corresponding trace from CHI over-expressing line 4-11. Levels of several of the kaempferol and quercetin conjugates identified in FIG. 2A were strongly elevated, and a number of new peaks were observed. The new compounds were identified as conjugates of quercetin; glucose-rhamnose-quercetin, and three other conjugates in which the sugars were not identified. FIGs. 2C and 2D show traces from the same tissue samples following ß-glucosidase digestion of the leaf extracts. Peaks a, b, e, f and g in FIGs. 2A and 2B have disappeared, and new peaks (j-n) have appeared, confirming the presence of ß-glucose linkages in peaks a, b, e, f and g.

Table 1 summarizes the levels of the various kaempferol and quercetin conjugates in control *Arabidopsis* and the three alfalfa CHI over-expressing lines: 4-9, 4-11 and 4-25. For the analysis, 4 g fresh weight of leaves was extracted twice with iced acetone and acetone:

methanol (1:1) sequentially, concentrated extracts were dissolved in 3 ml methanol, 20 μl of extract was injected for HPLC analysis. Over-expression of CHI resulted in an approximately 4-fold to 7-fold increase in total kaempferol and quercetin conjugates. Thus, genetic manipulation leading to over-expression of CHI results in large increases in the levels of antioxidant flavonoid derivatives in transgenic plants.

Table 1. Levels of flavonols in transgenic *Arabidopsis* lines expressing alfalfa CHI*

Lines	RGRQ	RRQ.	GRQ	Rutin	Q conjug.	RGRK	RRK	K cpnjug.	Total (μg/g F>W>)
Ctrl.	4.4	8.7	--	--	--	15.3	29.1	--	57.4
4-9	20.4	27.3	8.9	17.4	18.5	51.6	94.4	8.2	246.7
4-11	13.8	53.0	15.6	27.9	73.8	71.1	129.5	12.2	396.8
4-25	13.7	40.9	15.9	34.0	25.6	64.6	118.3	17.6	330.6

* R = rhamnose, G = glucose, Q = quercetin, K = kaempferol.

Example 3

Flavonoid/isoflavonoid production in transgenic *Arabidopsis* expressing both soybean CYP93C1v2 and alfalfa CHI

To test whether increasing flux into flavonoid biosynthesis by expression of alfalfa CHI would result in a corresponding increase in genistein production, IFS transgenic line 15b was crossed with line 4-11 harboring the alfalfa *CHI* transgene, and F_2 progeny plants screened for retention of *IFS* and *CHI* transgenes by PCR. Positive progeny were then analyzed by HPLC for content of genistein (after hydrolysis of leaf extracts) and flavonols. Homozygous 15b and 4-11 plants (T_4 generation) were included for comparison. FIG. 3A-3D show HPLC traces of leaf extracts from control (Ctrl; FIG. 3D), CHI 4-11 transgenic (CHI; FIG. 3B), IFS 15B transgenic (2-HIS15B; FIG. 3C), and CHI/IFS transgenic (CHI/2-HIS15b; FIG. 3A) progeny *Arabidopsis* plants. The extracts had been subjected to acid hydrolysis in order to hydrolyze all sugar conjugates and thereby simplify the HPLC traces. The results confirmed the elevated levels of kaempferol and quercetin in line 4-11 as compared to control plants, and the production of low levels of genistein in the IFS 15b line. One of the F_2 progeny lines expressing both transgenes appeared to contained higher levels of genistein than did 15b T_4 plants (Table 2). However, two F2 progeny of the cross contained lower flavonol levels than did line 4-11.

A larger number of F_3 progeny plants were next examined for isoflavone and flavonol levels, and *CHI* transgene product activity. The results confirmed the reduction in flavonol levels in plants producing genistein (Table 2). For most F_3 lines, levels of flavonol conjugates were between 13 and 70% of the average value for the 4-11 line. However, the data failed to confirm a relation between high genistein levels and elevated CHI activity. Two lines with highest genistein levels (3-3-1 and 3-4-1) had lost the *CHI* transgene through segregation, as determined by both PCR and the total lack of enzyme activity of crude extracts when supplied with isoliquiritigenin as substrate (Table 2). Furthermore, for the F_3 progeny that had retained both transgenes, genistein levels were, overall, no higher than in line 15b. Therefore, although over-expression of CHI results in increased flavonol production in *Arabidopsis*, the increase in flux through naringenin does

not result in increased isoflavone production, and isoflavone accumulation appears to cause a disproportionate decrease in flavonol levels in CHI over-expressing plants. This suggests competition and interference between the flavonol and isoflavone pathways.

Table 2: Genistein and flavonol levels in individual progeny of transgenic Arabidopsis expressing soybean IFS and/or alfalfa CHI*

Line	Generation	Alfalfa CHI activity (nmol/min/mg)	Quercetin (nmol/g FW)	Kaempferol (nmol/g FW)	Total flavonols (nmol/g FW)	Genistein (nmol/g FW)
Wild-type	T_4	0	32.4±5.9	285.8±35.7	318.2±36.7	0
CHI 4-11	T_4	49.6±3.0	79.6±16.4	475.3±77.8	555.0±84.5	0
IFS 15b	T_4	0	29.4±7.3	162.7±37.4	192.1±43.9	11.9±1.9
IFS/CHI-1	F_2	ND[b]	77.1±11.2	247.9±60.5	325.0±70.7	43.9±1.3
IFS/CHI-2	F_2	ND	23.0±9.1	216.5±50.8	239.5±56.4	9.6±3.1
IFS/CHI-3	F_2	ND	89.7±8.4	503.6±48.8	593.3±57.2	9.0±1.8
IFS/CHI 3-2-1	F_3	38.8±2.3	65.4±5.8	334.9±21.9	400.3±27.6	11.3±2.7
IFS/CHI 3-2-2	F_3	42.6±1.9	38.2±6.2	297.0±7.4	335.2±13.2	10.7±1.5
IFS/CHI 3-2-3	F_3	35.2±1.7	12.8±5.2	67.9±6.8	80.7±1.7	5.7±1.2
IFS/CHI 3-3-1	F_3	0	35.9±3.6	81.4±9.6	117.2±12.5	19.4±2.4
IFS/CHI 3-4-1	F_3	0	21.4±4.1	75.0±11.9	96.4±11.3	17.6±0.4
IFS/CHI 3-4-2	F_3	46.5±1.0	40.7±4.3	205.3±17.5	246.0±21.7	16.4±0.3
IFS/CHI 3-4-3	F_3	52.6±1.6	49.9±7.1	311.5±9.1	361.4±13.3	13.5±2.5
IFS/CHI 3-4-4	F_3	43.8±0.6	31.4±2.6	251.0±6.4	282.4±7.8	8.2±1.6
IFS/CHI 3-5-1	F_3	63.6±1.3	77.3±8.4	672.3±8.6	749.6±17	7.0±0.8

* For the analysis, plants were grown under 12 hr light/12 hr dark, ~150 μE light intensity, for 35 days. Data for T_4 plants are means ± SE from at least five replicate plants with 2-3 independent analyses for each plant. Data for individual F_2 and F_3 progeny represent triplicate analyses. ND, not determined.

Example 4

Production of Genistein in the *tt3/tt6* Mutant.

The *tt3/tt6* double mutant of *Arabidopsis* is impaired in expression of both flavanone 3-ß-hydroxylase (F3H), the entry point enzyme for flavonol/anthocyanin synthesis, and the downstream enzyme dihydroflavonol reductase (DFR) (Winkel-Shirley, 2001). *tt6/tt3* is in ecotype Landsberg Erecta. Soybean IFS was transformed into both wild-type Landsberg and *tt6/tt3* to determine the effects of a blockage in flavonol synthesis on genistein production. One confirmed transgenic *tt6/tt3* line was taken to the T_3 generation, and levels of flavonols and genistein determined by HPLC (FIG. 4B). Surprisingly, both kaempferol and quercetin were detected in all *tt6/tt3* lines, although, in the case of kaempferol, at levels at least one order of magnitude less than in wild-type Landsberg (FIGs. 4A, 4B, 4E). Expression of IFS in wild-type Landsberg resulted in low levels of genistein, similar to those in the Col-0 background (FIGs. 4D, 4F). However, *tt6/tt3* expressing IFS produced much greater amounts of genistein, with levels from 31-169 nmol/g fresh wt in three T_3 progeny plants (FIGs. 4B, F).

Arabidopsis thaliana is a member of the cruciferae, and therefore related to a number of vegetable and oil crops such as cabbage, sprouts, cauliflower, mustard and oil seed rape. Crucifers themselves contain health-promoting compounds such as certain glucosinolates (Faulkner *et al.*, 1998), and such species can be made even more "healthy" by incorporation of genes leading to accumulation of isoflavones. The results described herein provide proof of principle for a metabolic engineering strategy that should find wide usage in vegetable, grain and fruit species.

Example 5

Expression of *Medicago truncatula* IFS in alfalfa

The open-reading frame of *Medicago truncatula* IFS (MtIFS, TC45136) was cloned into the binary vector pCAMBIA 2300 under control of the double cauliflower

mosaic virus 35S promoter. This vector was transformed into alfalfa Regen SY-4D (Bingham, 1991) by *Agrobacterium*-mediated transformation of leaf discs according to standard procedures. Transgenic plants were obtained following somatic embryogenesis. Plants were subjected to PCR, DNA gel blot analysis, and RNA gel blot analysis to verify transformation.

Leaves of transgenic alfalfa plants were extracted in 80% methanol and analyzed for flavonoid content by HPLC (FIG. 5). To simplify the analysis, extracts were acid-treated to hydrolyze flavonoids to their aglycones. Control plants transformed with the "empty" binary vector were found to accumulate the flavone apigenin but no isoflavonoids were detected (FIG. 5A). Extracts of plants expressing MtIFS contained two additional peaks, which were identified as genistein and biochanin A by comparing UV spectra and retention times to authentic standards and by LC-MS analysis (FIG. 5B). Genistein levels were found to vary from 0-133 nmol/g FW in the leaves of 42 independent MtIFS-overexpressing lines (FIG. 5C).

In unhydrolyzed leaf extracts no free genistein or biochanin A was detected, but at least 5 peaks with UV spectra similar to these isoflavones were present in MtIFS-overexpressing line C22 compared to a control line (FIG. 6A, B). Alfalfa leaves have been shown to accumulate glucuronic acid conjugated flavones (Stochmal *et al.*, 2001a; Stochmal *et al.*, 2001b) and digestion of control leaf extracts with β-glucuronidase led to the appearance of free flavones including apigenin (FIG. 6D). However, β-glucuronidase digestion did not shift the five isoflavone peaks present in MtIFS-overexpressing leaves (FIG. 6C). These peaks were shifted by digestion with β –glucosidase to free genistein and biochanin A (FIG. 6E), indicating that these isoflavones were present as glucose rather than glucuronic acid conjugates. Peaks 1 and 4 were further identified as glucose-genistein and glucose-biochanin A, respectively by LC-MS analysis.

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